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INTRODUCTION

The general purpose of the present project is to evaluate flow cytometry as a means of detecting, enumerate and characterise bacteria harvested from air and water samples as part of a system for continuos monitoring of the environment with regard to potentially harmful organisms.

In this initial phase of the project we have, in accordance with the project proposal, concentrated on the aspect of detection and enumeration of bacteria, using as a model a variety of species grown in culture.

When bacteria are measured for monitoring purposes it is preferable to work with vital unfixed cells, partly to save time and partly because fixation of cells may cause aggregation, and also mask phenomena associated with vital functions, including the very aspect of vital versus dead. Thus, much of our effort has been directed towards development of staining procedures for unfixed samples. In order to establish such procedures, screening experiments with different fluorescent dyes were necessary. The emphasis has been primarily on the effects of different buffers and temperature as means of permeabilising the cell wall sufficiently to admit the dyes used, but not enough to facilitate efflux of macromolecules.

Flow cytometry (FCM) is a technique for measuring the fluorescence and the light scattering of individual cells in large numbers. The cells are measured as they flow in a fluid stream one by one through the focus of an intense light source. The fluorescence intensity (FL) is (usually) proportional to the cellular content of the fluorescent substance(s). Most FCMs can measure two or more fluorescence wavelength components simultaneously, thus facilitating the staining of cells with two or more different dyes.

The intensity of the scattered light (LS) yields information on the size, shape and structure of cells. Whereas the forward scattering, i.e. that detected at small scattering angles, is primarily a function of size the scattering to higher angles depends to a larger extent on cell shape and structure. Hence, the uptake of fluorescent dye in the cells can be correlated directly with their size and structure. The FCM can measure/detect fluorescence (FL) and light scattering of individual cells at a rate of several thousand cells per second. FCM has also the advantage that the measurements are not affected by the presence of free dye in the medium in which the cells are measured. This eliminates loss of dye from the cells during washing. It eliminates centrifugation which may cause aggregation and cell loss, and it thereby significantly shortens the time between incubation with dye and measurement.

FCM has been used extensively for research purposes of eucaryotic cells, but far less in studies of procaryotic cells, partly since most flow cytometers available must be pushed to their very limit of performance in order to yield data of adequate quality for bacterial analyses. However, the assay should have a significant potential also in such analyses.

METHODS

By means of a mercury based arc lamp FCM, we have developed and established conditions for measurement of bacterial cells. The fluorescence and light scattering of individual cells have been measured on an Argus flow cytometer (Skatron A/S, Norway), which is a forerunner of the current Bryte HS (BioRad, Inc., USA). The instrument is currently equiped with forward and large angle scattering and 3 fluorescence detectors. So far we have limited the measurements to one fluorescence parameter and forward light scattering.

We have been working mainly with Gram-negative bacteria, i.e. *Escherichia coli* and

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other species belonging to the *Enterobacteriaceae* group, as well as *Pseudomonas aeruginosa*. Species of Gram-positive bacteria have also been included, i.e. *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Streptococcus faecalis*, and *Bacillus cereus*. Most of the data have been based upon results obtained for ATCC and NCTC reference strains of the species mentioned, however, isolates from clinical samples have also been used. We have been working mainly with exponentially growing bacteria, but also with bacteria in stationary phase. Monodisperse, fluorescent polymer beads of diameter 1.5 μm have been run to standardise the measurement and ascertain proper function of the instrument at the beginning and end of each experiment. Each sample has been measured at a rate of up to 1500 cells/sec.

We have thus been working with several fluorescent dyes, mainly with nucleic acid binding dyes, i.e. ethidium bromide (EB), acridine orange (AO), mithramycin (Mi), and a combination of ethidium bromide and mithramycin (MiEB). Unfixed cells have been stained mainly with EB or AO, while mainly MiEB has been used for fixed cells. Cells for fixation were harvested in parallel to viable samples and centrifuged immediately at 17000 rpm for 3 min, washed in 10 mM Tris-buffer, resuspended in 10 mM Tris/ethanol 70 % (final concentration) and left overnight at 4 °C. The fixed cells were then washed once and resuspended in 10 mM Tris, stained on ice and analysed within 5 min at room temperature in the FCM. For the bulk of experiments preparation of unfixed samples always included immediate transfer of cells from the growth medium into various buffer solutions (PBS, Tris, or EDTA), storage at a given temperature for about 30 min, at which point dye was added and the cells analysed in the FCM within minutes. Initially, however, a wide range of measuring conditions were investigated to determine roughly the range of conditions that seemed appropriate.

RESULTS

In order to determine the optimal concentration of EB, fluorescence was measured versus EB concentration. The results are shown in Fig. 1. On the basis of these results EB was thus used in a concentration of 20 $\mu\text{g}/\text{ml}$, the corresponding value for AO and Mi was 50 and 100 $\mu\text{g}/\text{ml}$, respectively, in the subsequent experiments.

When working with unfixed bacteria, we experienced a great variation in cellular dye uptake. Thus, in most samples of Gram-negatives, the majority of the cells were hardly stained at all, while a minority exhibited a fluorescence close to that of fixed, i.e. fully permeabilized, control cells. In contrast, Gram-positive bacterial cells, were usually fully stained within minutes after addition of EB. Fig. 2 shows a typical two-parameter LS versus FL histogram of exponentially growing *E.coli* cells transferred to room temperature PBS, left for about 30 min, and stained with EB. The majority of these cells exhibit quite low fluorescence (Note the logarithmic scale of fluorescence), whereas a minority of the cells show more than hundred fold higher fluorescence, i.e. fluorescence being close to the corresponding value for fixed cells, which supposedly are fully stained (Fig. 3). Generally, cells with low fluorescence under these conditions were regarded as viable, whereas cells with high fluorescence were regarded as dead in accordance with the results of parallel vitality assays. This difference in dye uptake between vital and dead cells gives rise to a characteristic distribution of cells in the histograms, i.e. typically in two sub populations with widely different net dye uptake. It is important to note that with increasing time in room temperature buffers, fluorescence of the viable cells remained about constant within a factor of 2 even after several hours.

Acridine orange (AO) gave results generally similar to those obtained with EB, whereas mithramycin yielded significantly lower fluorescence intensities, supposedly due the fact that the fluorescence quantum yield of this dye is much below that of AO and EB. AO is approximately as bright as EB. However, we settled for EB for the subsequent experiments

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for two reasons: 1) Toxicity. AO is registered as mutagenic and potentially carcinogenic. 2) AO sticks quite efficiently to a large variety of materials, including the tubing (Teflon) of the sample system of the instrument. Hence, the system is difficult to clean, and the sticking dye may cause measuring artefacts.

An important observation is that with the present instrument the light scattering of individual cells was always easily discernible from the background. This means that under the present conditions light scattering alone may be sufficient to detect and enumerate bacteria. The signal to noise ratio in the present measurements also implies that this will hold true even for much smaller cells than *E. coli*. On the other hand, this is not to say that light scattering detection will be sufficient under all circumstances. It is to be expected that environmental samples may contain large amounts of cellular debris and other particulate matter, including particles within the same size range as bacteria. In such cases fluorescence detection of dyes particular to bacteria, or at least living organisms, may be a prerequisite for detecting and counting the bacteria content of the samples.

Fig. 4 shows a histogram obtained for a Gram-positive bacteria, namely *S. faecalis*. These cells were harvested at exponential growth, transferred to PBS at room temperature, left for 30 min, stained with EB, and analysed after few minutes. The data demonstrate that the fluorescence of these cells was close to that of fixed control cells (Fig. 3). Thus, the dye permeability of these cells was so high that they could be fully stained with EB without any further permeabilization.

The low dye uptake in viable Gram-negative bacterial cells was found to be associated with efflux of the probes. Thus, previous studies have demonstrated the presence in *E. coli* (and other bacterial species) of an efflux system for several toxic organic compounds, including phosphonium ions, ethidium, and derivatives of acriflavine. The efflux activity has been found to be dependent on the electrochemical potential across the cytoplasmic membrane. This appears to be at least part of the reason for the large difference in the staining of vital and dead gram-negative cells.

Accordingly, we spent a significant part of our time to study this phenomenon in some detail in order to determine optimal staining conditions for gram-negative bacteria in particular. These experiments suggest that we may succeed in our main aim of the first part of this project, namely to determine conditions that will allow essentially complete staining of the DNA of vital cells. The results will be reported in detail in a forthcoming report.

CONCLUSIONS

Among the dyes we have studied so far ethidium bromide may appear as the best candidate. Acridine orange presents problems with toxicity as well binding to the sample system of the instrument. The fluorescence of mithramycin is weaker than that of AO and EB. On the other hand this dye is highly DNA specific in contrast to both AO and EB which bind also to RNA.

However, the efficient efflux of these dyes from vital gram-negative bacteria makes them less than ideal. On the other hand, since this efflux pump depends on metabolic activity, these dyes may be used to distinguish between live and dead cells. It should be noted here the present flow cytometer facilitates simultaneous measurement of two different dyes, which is to say that cells may be characterised on the basis of two different functions. This aspect will be taken up at a later stage of the project.

We are also planning to look at other types of dyes, including both DNA specific ones and dyes binding to the cell wall. One purpose of this will be to search for dyes which are not affected by the efflux pump of the cells.

The light scattering sensitivity of the Argus flow cytometer that we have been using is fully sufficient to detect bacteria with adequate signal to noise ratio. Under some conditions, therefore, light scattering alone may be used to detect and count bacteria without the need for the use of fluorescent dyes. In the present measurements we have measured only forward scattering, that is scattering at low scattering angles. As noted above the instrument can measure separately light scattering to higher scattering angles. Previous studies have shown that simultaneous measurement of these two separate scattering components may facilitate distinction of bacteria having the same size, and therefore the same forward scattering signal, thus greatly increasing the resolution of the instrument with regard to different species of bacteria. This facility may also be useful to distinguish cells from other types of particles likely to be present in environmental samples, such as silicates. This aspect is to be studied further later in the project.

The efflux of dye from vital gram-negative cells is a major complication. We have found staining conditions which appear to overcome this problem without requirement for fixation or washing. These results will be detailed in a forthcoming report.

Finally, it should be noted that by means of the procedures described above, we are able to count bacteria flow cytometrically. These results are in good agreement with cell numbers measured by standard procedures, i.e. counting of colony forming units.

Figure legends.

Fig. 1. EB fluorescence of *E.coli*, harvested during exponential growth, fixed in 70 % ethanol, washed and stained with EB, plotted versus the staining concentration of EB.

Fig. 2. Fluorescence versus light scattering histogram of *E.coli* cells harvested. The major peak to the left in the histogram reflects viable cells, while the minor peak to the right reflects dead cells. Note that the fluorescence (FL) is on a logarithmic scale while the light scattering (LS) is on a linear scale.

Fig. 3. Fluorescence versus light scattering histogram of ethanol-fixed *E.coli* cells.

Fig. 4. Fluorescence versus light scattering histograms of *S.faecalis* cells harvested in exponential growth and transferred immediately to PBS at room temperature. After 30 min in buffer, EB was added, and the cells were measured within 10 min in the FCM. Note that fluorescence is significantly higher than the fluorescence obtained for *E.coli* cells (Fig. 1).